REMARKS

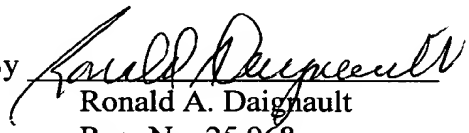
A new Abstract page is supplied to conform to that appearing on the publication page of the WIPO application, but the new Abstract is typed on a separate page as required by U.S. practice.

The specification was amended to rectify certain clerical or ministerial errors in the preparation of the original application.

Respectfully submitted,

SCHLIEVERT, Patrick M. et al.

By their attorneys,
MERCHANT & GOULD P.C.
3100 Norwest Center
Minneapolis, Minnesota 55402
(612) 332-5300

By 
Ronald A. Daignault
Reg. No. 25,968

sequence coding for a mutant SPE-C toxin having one change in amino acid sequence. This first PCR product then served as the template DNA to generate a second PCR product with two changes in amino acid sequence compared with a protein having wild type SPE-C activity. The first PCR product was the template DNA combined with a second internal primer coding for a change in amino acid at a second site. The second internal primer was also combined with the 5' and 3' flanking primers to form a second PCR product. The second PCR product was a DNA sequence encoding a mutant SPE-C toxin with changes at two sites in the amino acid sequence. This second PCR product was then used as a template in a third reaction to form a product DNA sequence encoding a mutant SPE-C toxin with changes at three sites in the amino acid sequence. This method is utilized to generate DNA sequences encoding mutant toxins having more than one change in the amino acid sequence.

An alternative method to prepare DNA sequences encoding more than one change is to prepare fragments of DNA sequence encoding the change or changes in amino acid sequence by automated synthesis. The fragments are then subcloned into the wild type SPE-C coding sequence using several unique restriction sites. Restriction sites are known to those of skill of the art and are readily determined from the DNA sequence of a wild type SPE-C toxin. The cloning is done in a single step with a three fragment ligation method as described by Revi et al. Nucleic Acid Res. 16: 1030 (1988).

EXAMPLE 6

Evaluation of Single and Double Mutants of SPE-C

Serine Mutants

Three single amino acid mutants of SPE C were made: a) Y15S in which tyrosine at position 15 was changed to serine, b) Y17S in which tyrosine at position 17 was changed to serine, c) N38S in which asparagine at position 38 was changed to serine. Two double amino acid mutants of SPE C also were made: a) Y15S/N38S, b) Y17S/N38S. All mutants were constructed by use of the Quik Change method (Stratagene, La Jolla, CA) with the *speC* containing plasmid pUMN521 as template. pUMN521 contains the SPE C gene (*speC*) in pUC13 (Goshorn et al.).

The single amino acid mutant proteins were produced in *Escherichia coli* in 100 ml cultures. After growth in the presence of 50μg/ml ampicillin, the *E. coli* cultures were treated with 400 ml -20 °C ethanol to lyse cells and precipitate SPE C mutant proteins. pUMN521 in *E. coli* was treated comparably for use as a positive control. The precipitates were collected and restored to 1 ml. Toxin concentrations were estimated to be 25μg/ml.

Wild type SPE C from pUMN521 and the three single amino acid mutants were evaluated for capacity to induce rabbit splenocyte proliferation over a toxin dose range of 0.25 to $2.5x10^{-5}$ or $2.5x10^{-6}$. As indicated in Figure 7, the Y15S and N38S mutants were approximately one half as mitogenic as the wild type. The Y17S mutant was essentially nonmitogenic (Figure 8).

The double mutants Y15S/N38S and Y17S/N38S were also tested for ability to stimulate rabbit splenocytes compared to wild type toxin (Figure 9). Both mutants stimulated rabbit splenocytes only to one-fourth that seen by comparable amounts of wild type toxin.

Both double mutants were also tested for capacity to enhance endotoxin shock. Three rabbits/group were challenged intravenously with 5μg/kg of mutants or wild type toxin. After 4 hours, the same animals were challenged with 10μg/kg *Salmonella typhimurium* endotoxin (1/50 LD_{50}). Deaths were recorded over a 48 hour time period (Table 4). As indicated, neither double mutant caused lethality in the rabbits.

Table 4: Capacity of double amino acid mutants of SPE C to enhance rabbit susceptibility to endotoxin shock.

Treatment Protein	Number Dead / Total Rabbits tested
SPE C wild type	3/3
Y15S/N38S	0/3
Y17S/N38S	0/3

Note: In the study reported in Table 4, all rabbits were challenged intravenously with 5μg/kg protein and then 4 hours later with endotoxin (10μg/kg).

One week after challenge of the rabbits used in Table 4, the animals were euthanized and examined for gross tissue damage. All organs, including liver, spleen, kidneys, lungs and heart appeared normal. This is consistent with the lack of toxicity of the double mutants.

Three rabbits/group were also immunized with two weekly doses of 25μg of SPE C double mutants emulsified in Freund's incomplete adjuvant. The animals were then rested for 5 days. 0.5 ml of blood was collected from each animal and pooled for collection of Y15S/N38S and Y17S/N38S sera. The sera from these pools was compared to preimmune pooled serum by peroxidase based ELISA (Hudson and Hay reference) for antibodies against purified streptococcal derived wild type SPE C. Table 5 summarizes the results of the ELISA.

Table 5: ELISA antibody titers of rabbits immunized against Y15S/N38S and Y17S/N38S mutants of SPE C.*

	Sample tested	ELISA titer:
Y15S/N38S	Preimmune	<10*
	Immune	80
Y17S/N38S	Preimmune	<10
	Immune	80

*Sera to be tested for antibody were diluted 2-fold beginning at 1:10. The titer of antibody is the reciprocal of the last dilution that gave an absorbency at 490 nm of 0.1 or greater.

The immunized animals were then challenged with 5μg/kg of wild type SPE C and then 4 hours later 10μg/kg of *Salmonella typhimurium* endotoxin as a test for capacity to immunize against lethality. Table 6 indicates the animals were protected from challenge and were thus immune to SPE C.

Table 6: Challenge of Y15S/N38S and Y17S/N38S immune animals with wild type SPE C and endotoxin.

Rabbit Group	Number Dead/Total Tested
Nonimmune	2/2
Y15S/N38S immune	0/3
Y17S/N38S immune	0/3

Additional single amino acid mutants of SPE C were also prepared. These include residues in the three major domains that may be required for toxicity. These include the T cell receptor binding domain, the class II MHC binding domain, and residues along the back of the central diagonal alpha helix. The residues changed and the effect of the mutation on T cell miotgenicity are listed in Table 7.

Table 7: Efect of mutants of SPE C on T lymphocyte mitogenicity and lethality

Mutant	Biological Activity	
	Mitogenicity[a]	Lethality[b]
D12A	Not tested	0/2
H35A	100% of wild type	Not tested
N38D	Not Tested	0/2
K135D	50% of wild type	Not tested
K138D	62% of wild type	Not tested
Y139A	54% of wild type	Not tested
D142N	52% of wild type	Not tested

[a] Comparison made at 0.1μg/well dose.

[b] Number of rabbits that succumbed/total injected due to enhanced susceptibility to endotoxin; 2/2 animals that received wild type SPE C died.

Alanine Mutants

Two single amino acid mutants of SPE C were made: a) Y15A in which tyrosine at position 15 was changed to alanine and b) Y17A in which tyrosine at position 17 was changed to alanine. Two double amino acid mutants of SPE C also were made: a) Y15A/N38A and b) Y17A/N38A, in which N38A designates a mutant in which asparagine at position 38 was changed to alanine. All mutants were constructed by use of the Quik Change method (Stratagene, La Jolla, CA) with the *speC* containing plasmid pUMN521 as template. pUMN521 contains the SPE C gene (*speC*) in pUC13 (Goshorn et al.).

The single amino acid mutant proteins were produced in *Escherichia coli* in 100 ml cultures. After growth in the presence of 50μg/ml ampicillin, the *E. coli* cultures were treated with 400 ml -20 °C ethanol to lyse cells and precipitate SPE C mutant proteins. pUMN521 in *E. coli* was treated comparably for use as a positive control. The precipitates were collected and restored to 1 ml. Toxin concentrations were estimated to be 25μg/ml.

Wild type SPE C from pUMN521 and the two single amino acid mutants were evaluated for capacity to induce rabbit splenocyte proliferation over a toxin dose range of 0.25 to $2.5x10^{-5}$ or $2.5x10^{-6}$. The Y15A and Y17A mutants were essentially nonmitogenic (Figure 8).

The double mutants Y15A/N38A and Y17A/N38A were also tested for ability to stimulate rabbit splenocytes compared to wild type toxin. Both mutants stimulated rabbit splenocytes only to less than one-sixth that seen by comparable amounts of wild type toxin.

Each of the mutants was also tested for capacity to enhance endotoxin shock. Two rabbits/group were challenged intravenously with about 25μg/kg of mutants or 5μg/kg wild type toxin (derived from Group A Streptococcal strain T18P). Temperatures were monitored rectally (Table 8). Then the rabbits were challenged intravenously with 5μg/rabbit of Salmonella typhinurium endotoxin. Deaths were recorded over a 48 hour time period (Table 8). As indicated, neither double mutant nor the Y15A mutant caused lethality in the rabbits. Each of the mutants reduced fever.

Table 8 - Toxicity of Alanine Mutants Compared to Wild Type

SPE C	Pyrogenicity - Δ°C at 4 hr.	Dead/Total
Wild Type	1.65	2/2
Y15A	0.3	0/2[a]
Y17A	0.65	1/2
Y15A/N38D	0	0/2[a]
Y17A/N38D	1.25	0/2[a]

[a] Animals did not show signs of streptococcal toxic shock syndrome, but rather, remained healthy

Additional Mutants

Additional single amino acid mutants of SPE C were also prepared. These include residues in the three major domains that may be required for toxicity. These include the T cell receptor binding domain, the class II MHC binding domain, and residues along the back of the central diagonal alpha helix. The residues changed and the effect of the mutation on T cell miotgenicity are listed in Table 7.

Table 7: Efect of mutants of SPE C on T lymphocyte mitogenicity and lethality

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[a] Comparison made at 0.1μg/well dose.

[b] Number of rabbits that succumbed/total injected due to enhanced susceptibility to endotoxin; 2/2 animals that received wild type SPE C died.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.